To United States Patent and Trademark Office:

Re: U.S. Serial No. 10/522,110
Completion of PCT/CN2003/000609
Based on Chinese Patent Application No. 02125917.8

DECLARATION OF INVENTIOR

I, the undersigned, Xu Qishou, declare and say:

1. I am a citizen of the People's Republic of China and I reside in Beijing, P.R.C.
2. I am a professor at the Institute of Radiation Medicine, Academy of Military Science, People's Liberation Army, Beijing, China, and I have been doing researches relating to the art of the present invention for more than 30 years.
3. I am one of the co-inventors of Chinese Patent Application No. 02125917.8, filed on August 2, 2002, entitled "A Riboflavin Derivative and Its Manufacture and Uses", which is the priority application of the present U.S. patent application Serial No. 10/522,110.
4. I am familiar with the references cited by the examiner in the captioned application.
5. I am aware of and helped structure the following test conducted to assess the cytotoxicity of riboflavin ester derivatives.
6. The tests are described in detail in ANNEX II and I will refer to the results obtained through the test.
7. In my opinion, the presently claimed invention, as evidenced by the results shown in ANNEX II, offers clearly unexpected results of using 5'-lauric acid ester of riboflavin as opposed to those known in the art, especially 5'-capric acid ester and 5'-myrisitic acid ester of riboflavin.
8. In my opinion, the selection of the specific esterification degree (monoester), esterification site (5'-), and ester-forming carboxylic acid (lauric acid), as claimed and shown in ANNEX II, offers an unexpected technical effect of providing a substantially-reduced cytotoxicity.
9. I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made may jeopardize the validity of

the application or any patent issued thereon.

Respectfully submitted,

Qishou, Xu
Professor of Institute of Radiation Medicine, Academy of Military Medical Sciences, People's Liberation Army
Date: July 20, 2010

ANNEX II OF DECLARATION OF QISHOU XU FOR U.S. PATENT APPLICATION NO. 10/522,110

Object

To access the effects of various riboflavin ester derivatives on the growth of human embryo lung fibroblasts (HELF).

Compounds to be tested

Riboflavin-5'-laurate (*hereinafter referred to as "5'-laurate"*), having a purity of 99.3%.

Riboflavin-5'-monocaprate (*"5'-caprate"*), having a purity of >99%.

Riboflavin-5'-monomyristate (*"5'-myristate"*), having a purity of >98.5%.

(These compounds to be tested were synthesized by reacting riboflavin with lauric acid chloride, capric acid chloride, and myristic acid chloride, respectively, and purified by HPLC. Both the riboflavin and the acid chlorides are commercially available.)

The aforesaid compounds to be tested were dissolved into DMSO and diluted to desired concentrations with culture medium.

Cells to be tested

Human embryo lung fibroblasts (HELFs).

Method

Cells were inoculated onto the RPMI1640 medium containing 10% fetal bovine serum (supplemented with 100 ku/L of penicillin and 100 ku/L of streptomycin). The culture vessel was placed in an incubator containing 5% of CO_2 at 37°C. The medium was refreshed every 2-3 days. The culture was digested with 0.25% of trypsin. Cells were passaged and collected.

The cells in logarithmic growth phase were formulated with the RPMI1640 medium containing 10% of fetal bovine serum to cell suspensions having the desired concentrations. The resultant cell suspensions were added into a 96-well plate with 3,000 cells (100 µl) per well. After incubation for 12-16 hrs, each well was fed with the compound to be tested with various concentrations. For the same concentration, 4-6 parallel wells were prepared. After incubation for 72 hrs, the supernatant was removed and each well was fed with 100 µL freshly formulated serum-free medium containing 0.5 mg/ml of tetrazolium blue (MTT). After incubation at 37°C for 4 hrs, the supernatant was removed. The resultant formazan was dissolved in 200 µL DMSO, mixed homogeneously in a mixer for 90s, and measured for absorptivity with an enzyme-labeling instrument at a wavelength of 570 nm.

Data Processing

The data were expressed by $\bar{x} \pm s$. The Inhibition = (OD-value of the control group - OD-value of the administration group)/OD-value of the control group ×100%。The data were plotted with MicroCal Origin software.

Experimental Instruments

Multiskan Ascent-Thermo Labsystems Enzyme-Labeling Instrument.

Results

The results indicated that: at the exposure concentrations of 120 and 240 μmol/L, the Inhibitions of 5'-laurate relative to HELF cells were 11.5% and 20.1%, respectively; the Inhibitions of 5'-caprate relative to HELFs were 29.8% and 33.0%, respectively; and the Inhibitions of 5'-myristate relative to HELFs were 24.4% and 26.0%. Please see Table 1 and Fig. 1 for details. It was indicated that the Inhibitions of 5'-myristate and 5'-caprate were higher than that of 5'-laurate.

Table 1. Effects of 5'-Laurate, 5'-Myristate, and 5'-Caprate of Riboflavin on Growth of HELF Cells

Compounds to be tested	Exposure Concentrations				
	120 μmol/L		240 μmol/L		0 μmol/L
	OD-Value	Inhibition	OD-Value	Inhibition	OD-Value
riboflavin-5'-laurate	0.602±0.087	11.5%	0.544±0.137	20.1%	
riboflavin-5'-myrisate	0.478±0.074	29.8%	0.456±0.073	33.0%	0.681±0.111
riboflavin-5'-caprate	0.515±0.084	24.4%	0.504±0.060	26.0%	

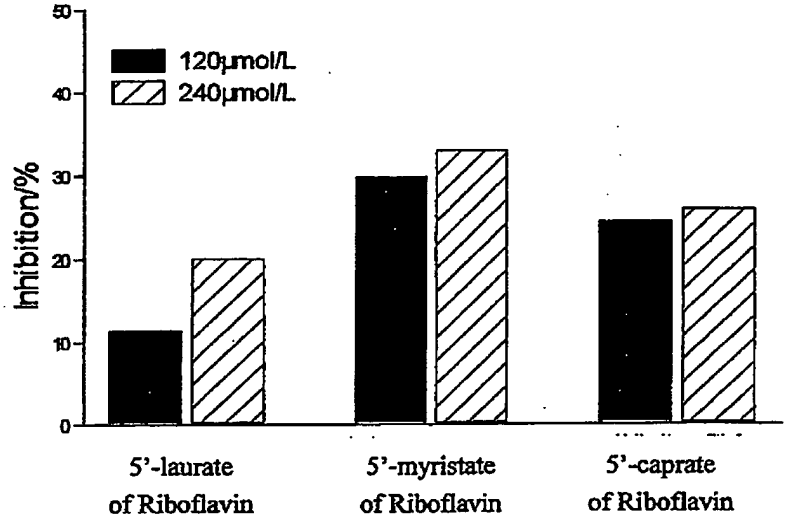

Fig. 1 Effects of 5'-Laurate, 5'-Myristate, and 5'-Caprate of Riboflavin on Growth of HELF Cells

Discussion

It can be concluded from the above-described experiments that at the same concentration, 5'-laurate of riboflavin as claimed in the present invention exhibit much less growth inhibition on HELF cells in comparison with 5'-caprate or 5'-myristate of riboflavin. That is to say, 5'-laurate of riboflavin has substantially reduced cytotoxicity compared with 5'-caprate and 5'-myristate of riboflavin if administered at the same concentration. Such unexpected result cannot be obviously derived from the common knowledge of the medical and pharmaceutical field, and thus the claimed compound, riboflavin-5'-laurate should be non-obvious.